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In vitro assessment of *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide, a DNA-intercalating antitumour drug with reduced sensitivity to multidrug resistance*

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Summary. The successful treatment of cancer requires the identification of new drugs with novel actions. *N*-[2-(Dimethylamino)ethyl]acridine-4-carboxamide dihydrochloride (DACA) is a topoisomerase II-targeted antitumour drug with curative activity against murine Lewis lung carcinoma. DACA was assessed for novel patterns of growth inhibition using normal and multidrug-resistant human cell lines. Cells were cultured in 96-well microtitre trays and tested against DACA and related topoisomerase-directed drugs, including amsacrine, etoposide and doxorubicin, and drug concentrations for 50% growth inhibition (IC_{50} or GI_{50} values) were determined. In a series of Jurkat leukaemia lines characterised as exhibiting atypical multidrug resistance, DACA was to a large extent capable of overcoming multidrug resistance exhibited towards the other topoisomerase-directed agents. DACA was also tested against the National Cancer Institute 60-tumour-specific cell-line panel (GI_{50} values ranging from 420 to 5,400 nM; mean, 2,100 nM) and against a series of primary cultures of surgically excised melanomas (IC_{50} values ranging from 60 to 1,600 nM; mean, 590 nM). DELTA values (deviations of logarithmic IC_{50} or GI_{50} values from the mean) were calculated and compared by correlation analysis. The standard deviation of DELTA values was found to be lower for DACA than for the other topoisomerase II-directed drugs amsacrine, etoposide, doxorubicin and mitozantrone in both the cell lines and the primary cultures. These lower standard deviations appear to have resulted from the reduced susceptibility of DACA to both P-glycoprotein- and topoisomerase II-mediated multidrug-resistance mechanisms occurring naturally in cell lines and primary cultures.

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Introduction

DACA (acridine carboxamide; NSC 601316; see structure in Fig. 1) was developed during a programme in the Auckland Cancer Research Laboratory to design and synthesise acridine derivatives with selective activity against solid tumours [2]. DACA binds to double-stranded DNA by intercalation [26] and is an efficient inducer of protein-DNA cross-links in cultured L1210 cells [23], consistent with its action as a poison of the enzyme topoisomerase. In contrast to the majority of topoisomerase II poisons, DACA is curative against the Lewis lung carcinoma in mice [2, 8], is active against a number of other solid tumours (unpublished data) and displays only moderate activity against experimental leukaemia [2]. DACA is currently undergoing preclinical toxicology and pharmacology testing, both in Auckland and in the United Kingdom, in preparation for clinical trial under the auspices of the Cancer Research Campaign, United Kingdom.

Multidrug resistance, the phenomenon whereby the development of resistance to one drug is accompanied by the simultaneous development of resistance to a variety of other, often structurally unrelated, drugs, is now recognised as an important clinical phenomenon [19] and is particularly relevant to topoisomerase II-directed antitumour drugs [15]. Several mechanisms of resistance have been identified, involving drug transport [7], alterations in the amount and/or degree of phosphorylation of topoisomerase II [4, 6, 12, 22] and drug detoxification [5]. The second mechanism of resistance, involving qualitative and/or quantitative changes in the target enzyme topoisomerase II, is often termed atypical multidrug resistance. DACA can be distinguished from doxorubicin and etoposide because of its lack of susceptibility to P-glycoprotein-mediated resistance [23]. In the present study we extended these results to show that DACA exhibits reduced susceptibility to a series of four multidrug-resistant Jurkat leukaemia lines selected for resistance to amsacrine or doxorubicin. These lines are cross-resistant to a number of topoisomerase II-directed drugs but show little or no resistance to other classes of drugs; resistance is not reversed by

verapamil and P-glycoprotein is not overexpressed. Although anthracycline metabolism is altered in the resistant cells, this is not thought to be the mechanism of resistance. Resistance is not mediated by altered drug uptake, altered DNA binding or altered DNA repair. Amsacrine stimulated the formation of protein-DNA cross-links in resistant lines to a much lower extent than in the parental lines. Taken together, these data suggest that the lines display atypical multidrug resistance [25].

The role of multidrug resistance in determining the sensitivity to topoisomerase II-directed agents of cell lines and primary cell cultures derived from human tumours is currently of great interest. A series of cell lines developed from a range of human tumour types presently forms the basis for a major drug-discovery programme sponsored by the United States National Cancer Institute (NCI) [1, 17]. The susceptibility of these lines to multidrug resistance is known to vary considerably, thus contributing to the large variation in the growth-inhibitory potency of susceptible drugs [27]. If DACA is capable of overcoming two multidrug-resistance mechanisms simultaneously, one might expect the range of such inhibitory concentrations to be reduced. We have tested this hypothesis using data from the NCI panel as well as data from a series of melanoma primary cultures derived in our laboratory [16]. The results highlight unique features of DACA as compared with existing clinical topoisomerase-directed agents.

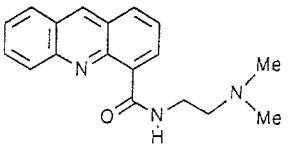

Fig. 1. Structure of DACA (free base)

Materials and methods

Materials. DACA (dihydrochloride salt) was synthesised in this laboratory [2], and amsacrine isethionate, obtained from the Parke-Davis Division of the Warner-Lambert Company (Ann Arbor, Mich., USA), were dissolved in 50% (v/v) aqueous ethanol to make stock solutions of 2–5 mmol/l and were stored at –20° C. Other cytotoxic drugs either were available from the NCI repository [17] or were obtained as previously described [10, 16].

Cells and tissue. The multidrug-resistant Jurkat lines were obtained from Dr. K. Snow and Dr. W. Judd, Department of Cellular and Molecular Biology, University of Auckland. Melanoma tissue was obtained from patients with pathologically confirmed metastatic and recurrent melanomas under Auckland Hospital Ethical Committee guidelines. Cells were released by digestion of tissue (at 50 mg ml^{-1}) with collagenase (1 mg ml^{-1}) and DNAase (50 μg ml^{-1}) under continuous stirring at 37° C for 1–2 h and were cultured as previously described [16].

Chemosensitivity assays. Tumour cell lines were cultured in 96-well plates. Growth of the leukaemia lines was assessed by IC_{50} values (defined as growth, indicated by staining or thymidine incorporation, corresponding to 50% of that of the control cultures) using (4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium (MTT) staining [18]. Growth of NCI cell lines was assessed by GI_{50} values (IC_{50} values corrected for time zero [17]) using sulphorhodamine B staining [24]. Growth of the primary human tumour material was assessed by IC_{50} values using [^{3}H]-thymidine incorporation [16]. Primary tumour material was cultured in 96-well plates in which the wells were coated with agarose for selective inhibition of the growth of normal cells [16]. Primary cultures were incubated at 37° C in sealed Perspex boxes (CBS Scientific, Del Mar, Calif., USA) containing a humidified atmosphere of 5% CO_2 and 5% O_2 in nitrogen for 7 days. 5-Methyl-[^{3}H]-thymidine (20 Ci $mmol^{-1}$; 0.04 μCi per well), thymidine and 5-fluorodeoxyuridine (each at final concentrations of 0.1 μM) were added in medium to cultures (20 μl per well) at 24 h before termination of the cultures. Cells were aspirated onto glass-fibre filters using a multiple automated sample harvester (LKB Wallac OY Beta Harvester, Skatron A/S, Norway). The filter discs were washed for 15 s with water and dried, and the amount of tritium retained was quantitated by liquid scintillation. DELTA values were determined for groups as deviations of logarithmic IC_{50} or GI_{50} values from the mean, with positive DELTA values representing higher drug sensitivity. Standard deviations of DELTA values were expressed in log_{10} units. DELTA values were compared using Pearson correlation coefficients. Resistance factors were defined as the ratios of IC_{50} values between the resistant line and the parent line. Statistical evaluation was performed using either standard NCI programmes, RS/1 software (BBN Research Systems, Cambridge, Mass., USA) or Sigmaplot (Jandel Scientific, San Rafael, Calif., USA).

Results

Normal and multidrug-resistant human leukaemia panel

The effect of DACA on the growth of cultured cells was assessed by continuous drug exposure. DACA inhibited the growth of two human Jurkat T cell leukaemia lines, one diploid (L) and the other tetraploid (B1), at IC_{50} values of 380 nM each. IC_{50} values for other human leukaemia cell lines ranged from 290 to 760 nM (CEM-CCRF, 410 nM; MOLT-4, 290 nM; Daudi, 400 nM; Raji, 370 nM, U937, 590 nM; HL-60, 620 nM; K-562, 760 nM). Four multidrug-resistant cell lines developed from L and B1 by in vitro exposure to increasing concentrations of doxorubicin (L_D and $B1_D$) or amsacrine (L_A and $B1_A$) [11, 25] were tested.

DACA was compared with six other drugs, including the four topoisomerase II poisons doxorubicin, mitozantrone, etoposide and amsacrine. Resistance factors for the topoisomerase II poisons were consistently higher for than those for DACA (Table 1). In contrast, the topoisomerase I poison camptothecin showed no cross-resistance, and the

Table 1. Resistance factors of drug-resistant Jurkat leukaemia sublines

	Doxo-rubicin	Etopo-side	Amsa-crine	Mito-zantrone	DACA	Campto-thecin	Vincris-tine
L_A	3.8	11	130	42	2.0	1.0	1.5
L_D	16	93	110	160	2.5	0.97	3.6
$B1_A$	11	22	240	59	3.9	0.48	2.0
$B1_D$	15	83	8.8	8.4	1.9	0.86	10

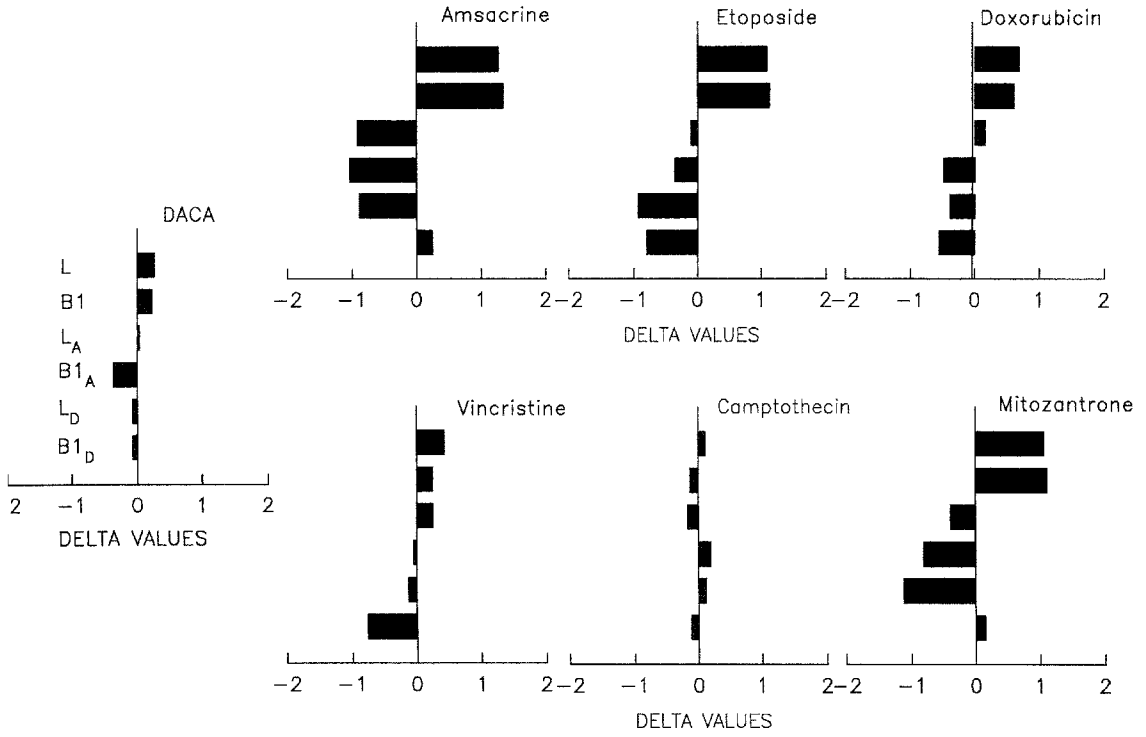

Fig. 2. Comparison of DELTA values plotted in log mean graph format for DACA, amsacrine, etoposide, doxorubicin, vincristine, camptothecin and mitozantrone using a panel of sensitive and multidrug-resistant Jurkat leukaemia lines. From top to bottom, *bars* represent L (parental), B1 (parental), L_A, $B1_A$ (resistant to amsacrine), L_D and $B1_D$ (resistant to doxorubicin)

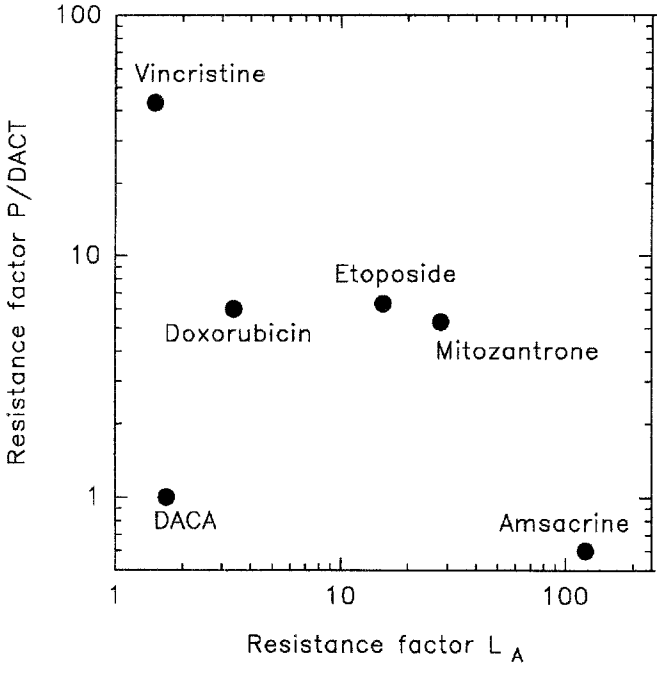

Fig. 3. Comparison of DACA with vincristine, doxorubicin, etposide, mitozantrone and amsacrine. Resistance factors are shown for a P-glycoprotein-positive (transport) multidrug-resistant P388 leukaemia line (P/DACT) and a Jurkat line exhibiting atypical (topoisomerase II) multidrug resistance (L_A)

mitotic inhibitor vincristine showed a different pattern of resistance, with the $B1_D$ line having the highest resistance (Table 1). To provide a visual comparison of the patterns of resistance, we plotted DELTA values [20]. Differences in bar lengths were used as a measure of relative resistance, and DACA clearly showed a pattern distinct from that of doxorubicin (Fig. 2).

As a second method of comparing agents, resistance factors for one of the cell lines were plotted against those of another. Since all four Jurkat lines exhibit predominantly "altered topoisomerase" resistance [11, 25], the resistance factors for one of these (L_A) was plotted versus the resistance factors for a P-glycoprotein-positive multidrug-resistant P388 leukaemia line (P/DACT) that exhibits transport resistance [3]. Vincristine was included as a drug that is susceptible to transport resistance but does not act on topoisomerase. In comparison with the clinical topoisomerase II agents, DACA was unique in its ability to overcome two different multidrug-resistance mechanisms (Fig. 3).

NCI human tumour-cell-line panel

DACA was also compared with three other topoisomerase II agents using a panel of cell lines encompassing a number of tumour types. The mean GI_{50} value for DACA was 2,100 nM as compared with 520 nM for amsacrine, 21,000 nM for etoposide and 140 nM for doxorubicin. The results were expressed as DELTA plots and compared with corresponding plots for three other topoisomerase II poi-

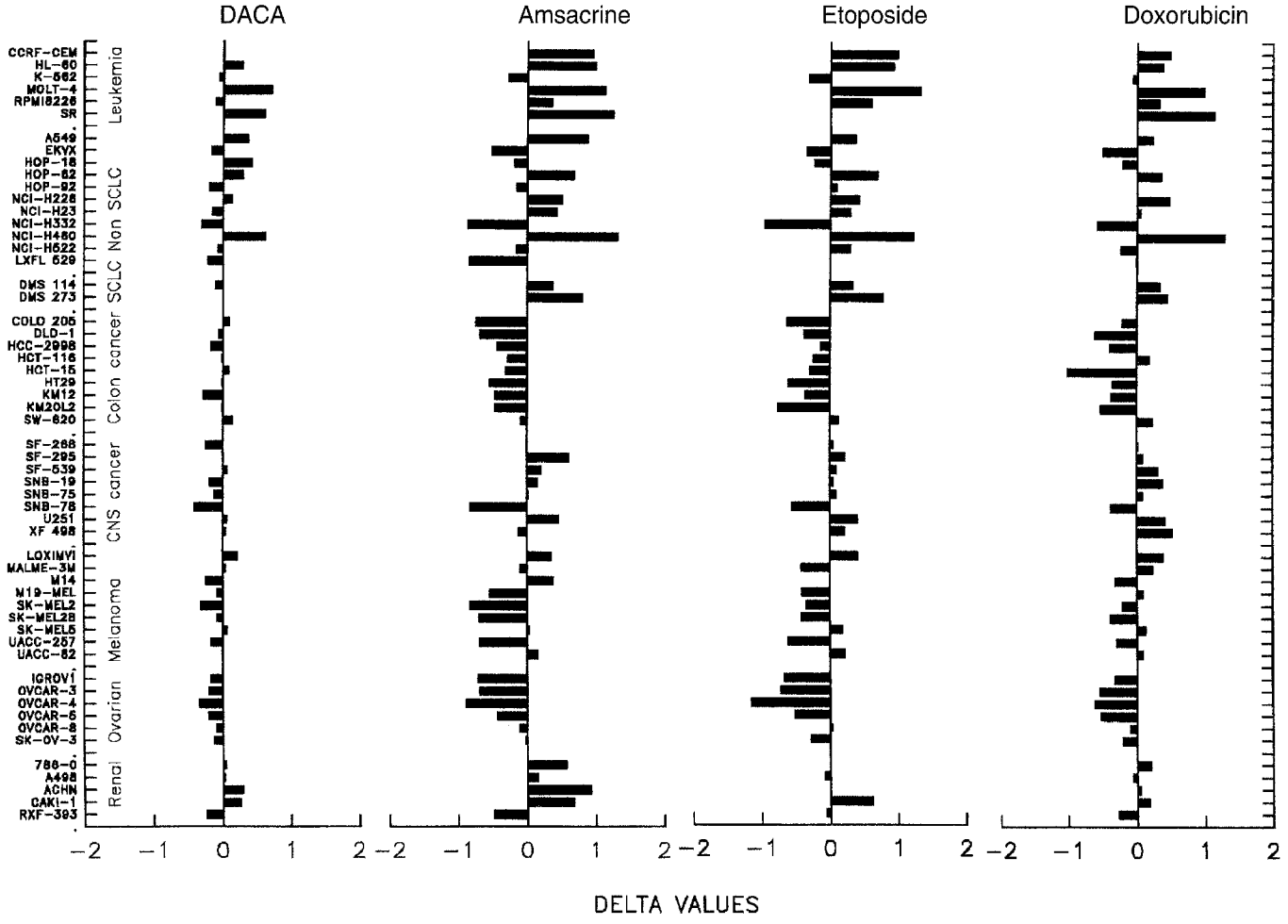

Fig. 4. Comparison of DELTA values plotted in log mean graph format for DACA, amsacrine, etoposide and doxorubicin using the NCI cell-line panel

sons (Fig. 4). The standard deviation of DELTA values was considerably smaller for DACA (0.24 units) than it was for amsacrine (0.61 units), etoposide (0.55 units) or doxorubicin (0.44 units). A comparison of the correlation coefficients for these drugs as well as mitozantrone and camptothecin indicated that DACA was related only distantly to the other topoisomerase-directed drugs (Table 2).

Primary melanoma-cell culture panel

DACA was compared with other drugs in a series of 12 primary melanoma cultures. Tissue was excised from human malignant melanomas and cultured using a modified 96-well assay system in which the cells were cultured on agarose and assayed for proliferation as previously described [16]. The mean IC_{50} value for DACA was 590 nM as compared with 128 nM for amsacrine, 2,200 nM for etoposide and 56 nM for doxorubicin. The values were lower than the respective melanoma cell-line values for these agents as tested using the NCI panel (assayed by protein staining: 2,300, 600, 16,000 and 87 nM, respectively). However, these differences were thought to reflect differences in the method of assessment of proliferation, since control experiments using MM96 and FME human melanoma cell lines assayed by protein staining with methylene blue [9] provided IC_{50} values for these drugs that ranged from 1.2- to 4.8-fold (data not shown) those reported for thymidine incorporation [16]. DELTA values for DACA, amsacrine, etoposide and doxorubicin were calculated (Fig. 5). As for the NCI cell lines, the standard deviation of DELTA values was smaller for DACA (0.39 units) than for amsacrine (0.54 units), etoposide (0.66 units) or doxorubicin (0.63 units).

Discussion

The unique relationship of DACA to existing clinical agents can be conveniently visualised when cross-resistance patterns are plotted in two dimensions (Fig. 3). Resistance factors for the Jurkat L_A line, which displays altered topoisomerase multidrug resistance [11, 25] is plotted in one dimension, whereas resistance factors to a P-glycoprotein-positive multidrug-resistant line are plotted in the other. As expected, vincristine is susceptible only to the latter resistance mechanism. Amsacrine is particularly susceptible to topoisomerase II-mediated resistance but, as

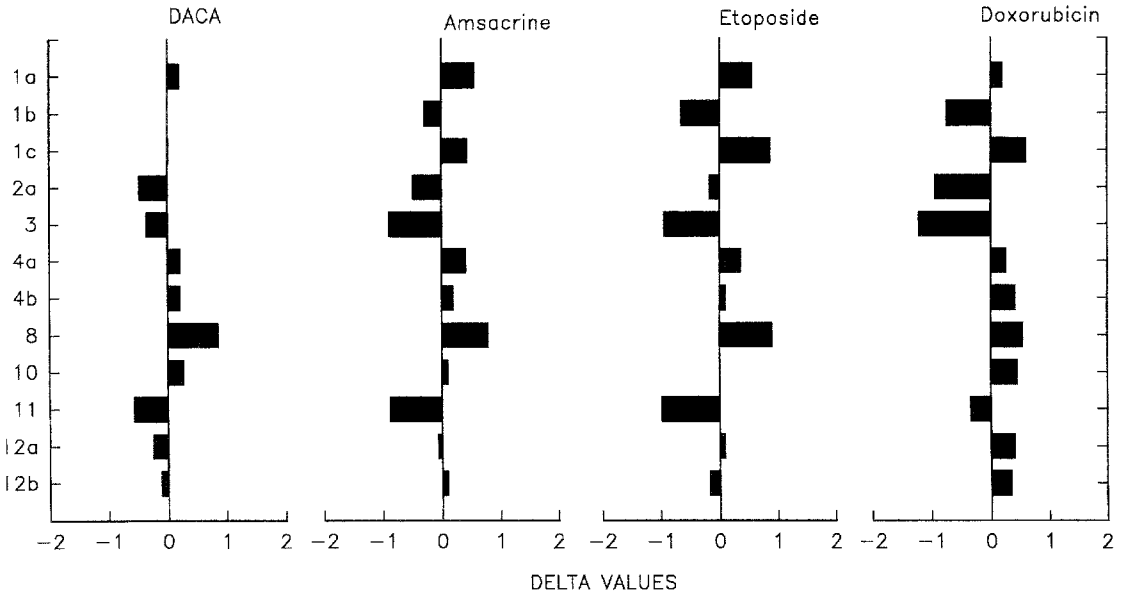

Fig. 5. Comparison of DELTA values plotted in log mean graph format for DACA, amsacrine, etoposide and doxorubicin using a number of primary cultures of biopsied tumour material. Numbers 1 a–12 a represent tumour-sample code numbers as reported previously [16]

previously found for both the murine P/DACT line [3] and the P-glycoprotein-positive, vinblastine-resistant human leukaemia line [21], is minimally susceptible to P-glycoprotein-mediated resistance. Doxorubicin, etoposide and mitozantrone are susceptible to both mechanisms, whereas DACA is refractory to both. It is noteworthy that DACA is nonetheless susceptible to the resistance mechanism associated with non-cycling cell populations, whereby topoisomerase II activity is reduced [8].

The hypothesis that the simultaneous reduction of susceptibility to two multidrug-resistance mechanisms would result in a reduced range of growth-inhibition values in unselected cell lines was tested using the NCI panel of cell lines. GI_{50} values for DACA cover a 13-fold range for the NCI panel as compared with a 120-, 270- and 200-fold range, respectively, for amsacrine, etoposide and doxorubicin (Fig. 4), consistent with this hypothesis. Although GI_{50} or IC_{50} values as determined by protein staining may be higher than those obtained by MTT staining or thymidine incorporation, the ranking obtained is very similar ([10, 14]; unpublished results), validating the use of different end points for comparison. The patterns of DELTA values for amsacrine, etoposide and doxorubicin show greater similarities with each other than does each with DACA, as demonstrated by correlation matrix analysis (Table 2). Comparison of DACA with other compounds in the NCI data base indicates that DACA is not closely related to any other compound (results not shown). The dissimilarity of the patterns of camptothecin and DACA (Fig. 2; Tables 1, 2) as well as the ability of DACA to kill cells at all stages of the cell cycle [8] also argue against the possibility that DACA acts on topoisomerase I. One of the goals associated with the use of the NCI cell-line panel is to identify compounds with large, tissue-specific differences in toxicity (i.e. large DELTA values). DACA would not have been identified as being of interest using this criterion, although it would have been highlighted as a compound of interest because of the lack of correlation of DELTA values with those of existing agents.

The hypothesis that a reduction in susceptibility to two multidrug-resistance mechanisms would result in a reduced range of growth inhibition in primary cultures was tested using cultures derived from surgical melanoma specimens. Melanoma is not sensitive to existing topoisomerase II-directed agents, but the choice of melanoma tissue was made because of the availability of a semi-automated culture method and the existence of comparative IC_{50} data for a number of other drugs [16]. IC_{50} values

Table 2. Correlation matrix for GI_{50} values in the NCI tumour-cell-line panel

Doxorubicin	1.00						
Etoposide	0.88	1.00					
Amsacrine	0.79	0.86	1.00				
Mitozantrone	0.77	0.80	0.83	1.00			
DACA	0.62	0.58	0.72	0.49	1.00		
Camptothecin	0.43	0.64	0.71	0.59	0.42	1.00	
Vincristine	0.19	0.28	0.21	0.34	0.04	0.17	1.00
	Doxo-rubicin	Etopo-side	Amsa-crine	Mitozan-trone	DACA	Campto-thecin	Vin-cristine

for DACA cover a 27-fold range for the melanoma panel as compared with a 48-, 69- and 64-fold range, respectively, for amsacrine, etoposide and doxorubicin (Fig. 5), showing the same trend to a smaller extent.

In conclusion, the results demonstrate unexpected activity for DACA against multidrug-resistant cells. The lack of susceptibility of DACA to P-glycoprotein-mediated multidrug resistance may result from its lipophilic character [2], which allows extremely rapid uptake by tumour cells (Haldane et al., manuscript in preparation). Drug uptake may thereby occur at a rate exceeding that of P-glycoprotein-mediated drug efflux. The lack of susceptibility of DACA to topoisomerase II-mediated resistance may be related to changes in specific contacts between the drug and the topoisomerase, perhaps resulting from the lack of the anilino side chain possessed by amsacrine. The in vitro chemosensitivity profile of DACA, in combination with its novel pharmacodynamic properties [13], may provide an explanation for its high in vivo antitumour activity.

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